Microbial development in distillers wet grains produced during fuel ethanol production from corn (*Zea mays*)

R. Michael Lehman and Kurt A. Rosentrater

Abstract: Distillers grains are coproduced with ethanol and carbon dioxide during the production of fuel ethanol from the dry milling and fermentation of corn grain, yet there is little basic microbiological information on these materials. We undertook a replicated field study of the microbiology of distillers wet grains (DWG) over a 9 day period following their production at an industrial fuel ethanol plant. Freshly produced DWG had a pH of about 4.4, a moisture content of about 53.5% (wet mass basis), and 4×10^5 total yeast cells/g dry mass, of which about 0.1% were viable. Total bacterial cells were initially below detection limits (ca. 10^6 cells/g dry mass) and then were estimated to be $\sim 5 \times 10^7$ cells/g dry mass during the first 4 days following production. Culturable aerobic heterotrophic organisms (fungi plus bacteria) ranged between 10^4 and 10^5 CFU/g dry mass during the initial 4 day period, and lactic acid bacteria increased from 36 to 10^3 CFU/g dry mass over this same period. At 9 days, total viable bacteria and yeasts and (or) molds topped 10^8 CFU/g dry mass and lactic acid bacteria approached 10^6 CFU/g dry mass. Community phospholipid fatty acid analysis indicated a stable microbial community over the first 4 days of storage. Thirteen morphologically distinct isolates were recovered, of which 10 were yeasts and molds from 6 different genera, 2 were strains of the lactic-acid-producing *Pediococcus pentosaceus* and only one was an aerobic heterotrophic bacteria, *Micrococcus luteus*. The microbiology of DWG is fundamental to the assessment of spoilage, deleterious effects (e.g., toxins), or beneficial effects (e.g., probiotics) in its use as feed or in alternative applications.

Key words: biofuel, distillers grain, ethanol production, microorganisms, microbiology.

Résumé : Des résidus de distillation de grains sont coproduits avec l'éthanol et le dioxyde de carbone lors de la production d'éthanol combustible par le moulage à sec et la fermentation du maïs-grain, quoique l'on ait peu d'informations microbiologiques fondamentales relatives à ces produits. Nous avons réalisé une étude en double sur le terrain de la microbiologie des sous-produits de distillation de grains humides sur une période de 9 jours suivant leur production, dans une usine d'éthanol carburant industriel. Les sous-produits de distillation fraichement produits avaient un pH d'environ 4,4, une humidité de 53,5 % (sur la base du poids humide) et contenaient des levures à une densité 4×10^5 cellules totales/masses sec, dont environ 0,1 % étaient viables. Le nombre de cellules bactériennes total était initialement sous les limites de détection (ca. 10^6 cellules/g de matière sèche) et a ensuite été estimé à $\sim 5 \times 10^7$ cellules/g de matière sèche au cours de quatre premiers jours suivant la production. Le nombre d'organismes hétérotrophes aérobies cultivables (champignons et bactéries) s'échelonnait de 10^4 à 10^5 CFU/g de matière sèche durant les quatre premiers jours et le nombre de bactéries acido-lactiques augmentait de 36 à 10^3 CFU/g de matière sèche durant la même période. Après 9 jours, le nombre total de bactéries et de levures/moisissures a plafonné à 10^8 CFU/g de matière sèche alors que le nombre de bactéries acido-lactiques atteignait presque 10^6 CFU/g de matière sèche. Des analyses de la composition en phospholipides de la communauté ont indiqué que celle-ci restait stable au cours des 4 premiers jours d'entreposage. Treize isolats distincts morphologiquement ont été récupérés parmi lesquels 10 étaient constitués de levures et de moisissures de 6 genres différents, 2 étaient constitués de souches de la bactérie lactique *Pediococcus pentosaceus*, alors que la bactérie hétérophile aérobie *Micrococcus luteus* constituait un seul isolat. La connaissance de la microbiologie des sous-produits de la distillation est fondamentale à l'évaluation de la pourriture, des effets nocifs (e.g., toxines) ou bénéfiques (e.g., probiotiques) reliés à son utilisation dans l'alimentation ou dans des applications alternatives.

Mots-clés : biocarburant, sous-produits de la distillation, production d'éthanol, microorganismes, microbiologie.

[Traduit par la Rédaction]

Introduction

Industrial fuel ethanol production from corn (*Zea mays*) produces 3 main products in relatively equal proportions from the contemporary dry milling and fermentation of raw grain: ethanol, carbon dioxide, and distillers grains (Jaques et al. 2003). In 2005, US ethanol refineries (largely corn dry grind facilities) produced nearly 4 billion gallons (15.1 billion litres, about double of 2002; 1 gallon (US) =

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3.785 dm^3) and 9 million metric tons of distillers grains, the chief coproduct of fuel ethanol production (RFA 2006). This 9 million metric tons of distillers grains produced in 2005 is about triple the amount produced in 2001 (RFA 2006). As fuel ethanol production in the United States doubles again to at least 7.5 billion gallons in 2012 to meet the renewable fuels standard implemented in the 2005 US *Energy Policy Act*, a parallel increase in the coproduction of distillers grains will be realized (RFA 2006). Already in 2004, almost 2% of the volume of gasoline sold in the United States was ethanol (Davis and Diegel 2004). Energy demands, emerging technologies, and politics are converging to generate explosive growth in the fuel ethanol industry in a global energy revolution (Lyons 2003).

Energy and greenhouse gas budgets for fuel ethanol production have been recently updated and analyzed, and it is certain that the coproducts, distillers grains, figure significantly in the outcome of these calculations (Wang 2005; Farrell et al. 2006). The value of the distillers grains is a key factor in the economics of the biofuels industry. At present, distillers grains are primarily utilized as livestock feed, principally for dairy and beef production, and are the fastest growing commodity livestock feed material. A critical limitation for this feed material is the shelf-life of the distillers grains, particularly when wet, and the process and storage conditions that influence their preservation. Intensive drying of distillers grains prolongs product storage and enables more economical transport but consumes additional energy and adds cost to the product. As the production of distillers grains increases rapidly with expanding fuel ethanol production, preservation will be increasingly important along with the development of alternative uses for these materials (Rosentrater 2006).

Despite the current and predicted scale of distillers grains production associated with the modern corn-based, dry grind fuel ethanol industry, there is little published data on the microbiology of this important coproduct. Limited and poorly accessible industry and extension data generally indicate that spoilage is stimulated by molds and perhaps aerobic heterotrophic bacteria, but experimental details are sparse. Spoilage diminishes the palatability and nutritional value of the distillers grains and increases the potential for toxic effects. Although aflatoxins are known to survive the distillation process, screening of incoming grains controls the toxin levels in freshly produced distillers grains (Munkvold et al. 2005). The potential for mycotoxin production following distillers grains production is unknown. One publication was located that reported on the microbiology of distillers grains from wheat fermentation (beverage industry) (Pedersen et al. 2004). These coproducts are also used as a cattle feed, and it was shown that they contain substantial populations of lactobacilli, some of which may function as probiotics and enable increased use for swine feed.

Freshly produced distillers wet grains (DWG) should have low numbers of viable cells and activities, as they emerge from the ethanol plant having recently experienced elevated temperatures (65–80 °C), high ethanol concentrations, low pH (≤4.5), and exposure to selective antibiotics. Following production, surviving populations may grow and will be joined by colonizing populations from the local environment. Our study's objectives were to provide an initial assessment of the microbiology of freshly produced DWG and to document changes in the microbial populations during intervals following their production. Culture-independent (total cells, community phospholipid fatty acid (PLFA) profiles) and culture-dependent (culturable numbers with selective media) analyses were combined to provide an assessment of total microbial numbers and those microbial groups implicated in spoilage (molds, aerobic heterotrophic bacteria) or that have probiotic potential (some lactic acid bacteria). Morphologically distinct organisms were isolated and identified.

Materials and methods

Initial experimental setup and sampling

Distillers wet grains with solubles exited the ethanol processing plant (>100 million gallons per year plant in the upper Midwest USA) via a screw conveyer, which dropped them into one of several piles in an uncovered holding bunker. The DWG deposited in the bunker are distributed by a loader to semi-trucks that transport the DWG to local feedlots and dairies. For this study, 4 disinfected (washed with 10% sodium hypochlorite and thoroughly rinsed with sterile water) black rubber tubs (60 L) were filled with freshly deposited DWG and set uncovered adjacent to the holding bunker. Samples were collected at the following time intervals: time zero (initial), 2 days, 4 days, and 9 days. During this period of time (24 April 2006 – 3 May 2006), the average daily low and high temperatures were 3 and 15 °C, respectively. About 4.6 cm of precipitation (rain) was recorded during this time interval, largely in the last interval following the sampling on day 4. Windy conditions generally predominated. Prior to each sampling, including the initial, the DWG in each tub were thoroughly mixed with a sterile polypropylene scoop. Initial samples for all physical and compositional analyses were collected in 2 gallon plastic bags. Microbiological samples were aseptically collected and dispensed into sterile Whirlpak™ bags that were immediately placed on ice. Within 2 h of sample collection, sample analyses for culturable bacteria were initiated, subsamples for direct observation were preserved (2% formalin, 4 °C), and subsamples for community PLFA analysis were placed at –80 °C prior to processing.

Physical and chemical characterization

Initial samples (t = 0 days) of the DWG were physically and chemically characterized at the outset of the experiment. For each sample collected from each of the 4 tubs, 4 subsamples were analyzed (except for protein, fiber, and fat, which utilized only 2 subsamples). Moisture content was determined following the American Society of Agricultural Engineers' Standard S352.2 method (ASAE 2004) and pH was measured following the American Association of Cereal Chemists' Official Method 02-52 (AACC 2000). Compositional analysis consisted of that for protein (determined using the American Organization of Analytical Chemists International (AOAC) Official Method 990.03), fiber (following the AOAC Official Method 978.10), and fat (following the AOAC Official Method 920.39) (AOAC 2003).

Cell enumerations

Fixed (2% formaldehyde) suspensions of DWG were dispersed by blending with 0.1% sodium pyrophosphate (Balkwill and Ghiorse 1985), disrupted by sonication (50 W at 45 kHz, 10 min) with Tween 80 (0.05% final concentration), and settled by centrifugation (1000*g*, 10 min). For total bacterial cells, aliquots of settled suspension were filtered under vacuum onto 0.2 μm pore-size black polycarbonate membrane filters (Hobbie et al. 1977) with cellulose–acetate support filters. Cells concentrated on filters were stained with acridine orange (0.001%, 3 min), washed with sterile water, and then stained with 4′,6-diamidino-2-phenylindole (0.01%, 3 min), washed, dried, and mounted in immersion oil (Cargille FF) under a glass coverslip. Total bacterial cells were enumerated under epifluorescent illumination using a Leica DM LB2 microscope equipped with a 100× objective, 100 W mercury bulb, and filter sets for acridine orange (Chroma No. 41001) and 4′,6-diamidino-2-phenylindole (Chroma No. 31000). A minimum of 5 fields and 200 cells were counted, or 20 fields when 200 cells were not achieved. For yeast and mold cells, aliquots of the settled suspension were filtered under vacuum onto 0.8 μm pore-size black filters (AABG, Millipore) and stained with aniline blue (1% in M/15 K_2HPO_4, pH 8.9) (Koch et al. 1986; Tournas et al. 2001). Cells were enumerated with a 40× objective under epifluorescent illumination with ultraviolet excitation (Chroma No. 31000). Total numbers of cells are expressed on a per gram dry mass basis by accounting for moisture content.

Microbial composition of DWG by cultivation

Solid media and standard dilution spread-plating were employed to enumerate total colony-forming units (CFU) of lactic-acid-producing bacteria (LAB), aerobic heterotrophic organisms including bacteria, and yeasts and molds. Serial dilutions were made in sterile phosphate-buffered saline (PBS: 1.18 g Na_2HPO_4, 0.223 g $NaH_2PO_4{\cdot}H_2O$, and 8.5 g NaCl, per litre; pH 7.5) and triplicate plates were inoculated at 10^{-1} – 10^{-6} dilutions of sample for each media. LAB were enumerated on deMan–Rogosa–Sharpe agar (MRS; Oxoid) incubated under anaerobic conditions (Gaspak system) at 37 °C for 48 h. Yeasts and molds were quantified on dichloran rose bengal choramphenicol agar (DRBC; Oxoid), supplemented with 100 mg/L chloramphenicol (Tournas et al. 2001) and incubated in the dark at 23 °C for 7 days. Aerobic heterotrophs (bacteria plus fungi) were enumerated on plate count agar (PCA; Oxoid) after incubation in the dark at 23 °C for 7 days. Total numbers of CFU (average of 3 plates) are expressed on a per gram dry mass basis by accounting for the moisture content of the DWG.

Isolation and identification of cultured organisms

All morphologically distinct (size, color, form, margin, elevation, texture, surface appearance) colonies arising from the spread-plating of weathered distillers grains on the 3 media types were isolated by conventional methods. The resulting purified isolates were identified by colony and cellular morphologies, growth form (trypan-blue-stained molds), Gram stain, and biochemical testing using the API® 20C AUX™ test kit for yeasts, the API® 20A test kit for anaerobes, and a suite of traditional tests (Smibert and Krieg 1994) for the sole aerobic heterotrophic bacterium. To confirm the identity of the 2 LAB isolated on MRS agar, DNA was extracted (Wizard® Genomic Purification kit; Promega, Madison, Wisconsin, USA), and the nearly full length 16S rRNA gene was amplified (50 μL reactions containing the following (all from Promega unless otherwise noted): 0.025 U/μL GoTaq® DNA polymerase, 1× GoTaq® PCR buffer, 2 mmol/L $MgCl_2$, 0.2 mmol/L dNTPs, 0.5 μmol/L of forward and reverse primers, and 2% bovine serum albumin (Roche, Indianapolis, Indiana, USA); PCR conditions: 2 min initial denaturation at 95 °C; 30 cycles of 1 min denaturation at 95 °C, 1 min annealing at 55 °C, and 1.5 min elongation at 72 °C; 5 min final elongation at 72 °C using the primers 8F (5′-AGAGTTTGATCCTGGCTCAG-3′) and 1492R (5′-GGTTACCTTGTTACGACTT-3′) (Lane 1991). The PCR products were purified (Wizard® PCR Purification kit) and then sequenced at the Iowa State Sequencing Facility with the primers 8F, 530F (5′-GTGCCAGCMGCCGCGG-3′), and 1100F (5′-GCAACGAGCGCAACCC-3′) (Lane 1991). Nearly full length sequences were assembled within Bioedit and compared with the GenBank database using BLAST (Altschul et al. 1990).

Table 1. Physical and chemical properties of the distillers wet grains.

Property	Mean
Moisture content (%w.b.)	53.6 ± 0.3
pH	4.37 ± 0.01
Protein (%d.b.)	28.93 ± 0.25
Fiber (%d.b.)	6.64 ± 0.39
Fat (%d.b.)	12.44 ± 0.25
Ash (%d.b.)	5.14 ± 0.14

Note: Data are means and standard deviation for the 4 independent replicate bins. %w.b. denotes percentage calculated on a wet mass basis. %d.b. denotes percentage calculated on a dry mass basis.

Microbial composition of DWG by community PFLA analysis

PLFA analyses were performed on DWG samples using standardized methods by Microbial Insights (Knoxville, Tennessee, USA) (White et al. 1979). Biomass was estimated from the quantity of ester-linked PLFAs, and a structural community profile was generated based on the relative abundance of phospholipid classes (White et al. 1979).

Results and discussion

Physical and compositional characterization of DWG

The samples studied had moisture contents ranging from 53.0% to 54.0% on a wet basis (Table 1) and were therefore highly susceptible to rapid spoilage. A maximum moisture content of ~12% is generally recommended for feed products because this level minimizes transportation costs and is microbiologically stable (Beauchat 1981). Storage recommendations for DWG typically range between 4 and 7 days (Tjardes and Wright 2002). The DWG had an acidic pH (4.4) and levels of protein, fiber, fat, and ash that are typical of values obtained for distillers grains (Larson et al. 1993; Spiehs et al. 2002; Belyea et al. 2004).

Fig. 1. Total numbers of cells (bacteria and yeasts and (or) molds) per gram dry mass of distillers wet grains by direct enumeration at the 4 sampling points. Data are means of independent replicate bins (n = 4) with one standard deviation, excepting n = 1 and 3 for bacterial numbers at day 4 and at day 9, respectively. Bacteria numbers at time zero were below the detection limit (BD) of ca. 10^6 cells/g dry mass.

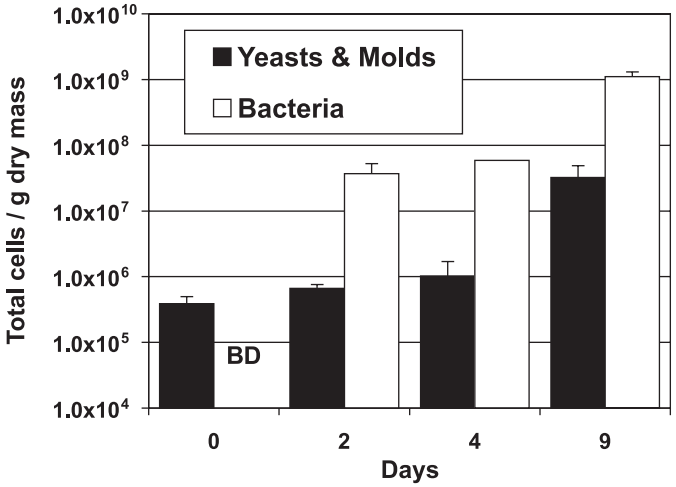
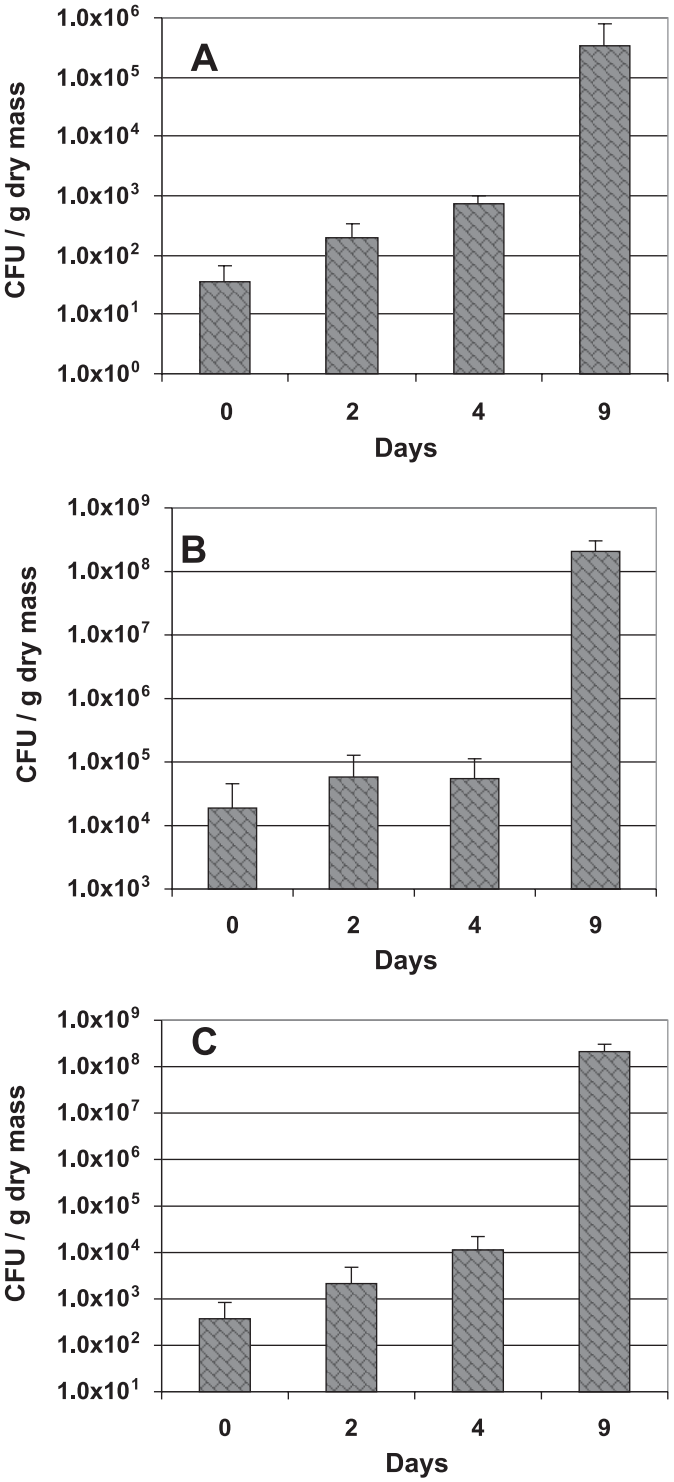


Fig. 2. Numbers of colony-forming units (CFU) per gram dry mass of distillers grains over time following discharge from the ethanol plant. Data are means with one standard deviation (n = 4). (A) Lactic-acid-producing bacteria; (B) aerobic heterotrophs (fungi plus bacteria); and (C) yeasts and molds.



Total fungal and bacterial cells

Initial direct counts of fungi averaged 3.9 × 10^5 cells/g dry mass in freshly produced DWG (0 days, Fig. 1) and were dominated by yeast cells. Total numbers of mold and yeast cells only rose slightly over the initial 4 days before much higher levels were observed at day 9 following production. The high levels of yeasts and molds enumerated at day 9 were probably an underestimate, since fungi tightly adhering to larger grain particles would not be included in the counts. Bacteria proved to be exceedingly difficult to enumerate in the DWG sample matrix; a number of approaches were attempted, with the best approach described in the Methods section. Initial bacterial cell counts were below detection (ca. 10^6 cells/g dry mass) and then were about mid-10^7 cells/g dry mass during the standard storage period. At day 9, high cell numbers were recorded (10^9 cells/g dry mass), indicating substantial growth. Bacterial cell numbers were underestimated owing to the deposition of particle-bound cells during sample processing and masking of cells during counting (Kepner and Pratt 1994). No correction factor was applied to account for this effect. Therefore, the numbers are best utilized relative to each other. No other published data on total cell counts of distillers grains are available for comparison with ours.

Culturable fungi and bacteria

Aerobic heterotrophs (bacteria and fungi) enumerated on PCA ranged between 10^4 and 10^5 CFU/g dry mass during the initial 4 day period before exploding to over 10^8 CFU/g dry mass at the 9 day sampling point (Fig. 2). Yeasts and molds enumerated on DRBC were about 3.8 × 10^2 CFU/g dry mass at day 0 and steadily increased to about 1.0 × 10^3 CFU/g dry mass at day 4. At the late day 9 sampling, there were over 10^8 CFU/g dry mass yeasts and molds. This number of culturable yeasts and molds exceeded the numbers recorded by direct observation (3.2 × 10^7 cells/g dry mass), presumably because of the technical issues described in the previous paragraph. Five different yeasts, including 3 *Candida* sp., were isolated on either PCA or DRBC from the weathered DWG (Table 2); none of them were strains of *Saccharomyces cerevisiae*, which apparently

Table 2. Isolate identities, medium used for recovery, and identification criteria.

Isolate	Identity	Group	Medium	Colony morphology	Cell morphology[a]	Gram stain	Biochemistry[b]
DG1-1	*Trichosporon asahii*	Yeast	PCA	X	X	X	X
DG1-2	*Cryptococcus* sp.	Yeast	PCA	X	X	X	X
DG1-3	*Candida* sp.	Yeast	PCA	X	X	X	X
DG1-4	*Alternaria* sp.	Mold	PCA	X	X		
DG1-5	*Micrococcus luteus*	Bacteria	PCA	X	X	X	X[c]
DG1-6	*Fusarium* sp.	Mold	PCA	X	X		
DG1-7	*Alternaria* sp.	Mold	PCA	X	X		
DG1-8	*Alternaria* sp.	Mold	PCA	X	X		
DG1-9	*Candida glabrata*	Yeast	DRBC	X	X	X	X
DG1-10	*Candida* sp.	Yeast	DRBC	X	X	X	X
DG1-11	*Penicillium* sp.	Mold	DRBC	X	X		
DG1-12	*Pediococcus pentosaceus*	Bacteria	MRS	X	X	X	X
DG1-13	*Pediococcus pentosaceus*	Bacteria	MRS	X	X	X	X

[a]Macroscopic and microscopic morphological characteristics were relied on for mold identification.

[b]API® test kits used as described in the text, except DG1-5.

[c]Biochemical tests used on DG1-5: lactose, glucose, mannose, and sucrose fermentation; indole formation, mixed-acid fermentation, Voges–Proskaeur, citrate utilization, sulfur reduction, motility, O-F glucose, O-F glucose with oil; urease, gelatinase, catalase, and oxidase activities.

did not survive the production process. Five different molds were also recovered using the 2 growth media. Three of the molds were *Alternaria* sp., one a *Fusarium* sp., and one a *Penicillium* sp. (the only mold recovered on DRBC). All 3 genera are widely known to colonize cereal grains and contain species that produce mycotoxins. No aflatoxin-producing *Aspergillus* spp. were recovered. Interestingly, only one aerobic heterotrophic bacterial species was recovered on PCA, *Micrococcus luteus*, a ubiquitous high G+C content Gram-positive organism that probably colonized the DWG from wind-blown soil particles.

There were about 3.6×10^1 CFU LAB/g dry mass in freshly produced DWG, and the numbers steadily increased during the initial 4 day period to about 10^3 CFU/g dry mass and then to about 10^6 CFU/g dry mass at 9 days. The 2 morphologically distinct LAB colonies (one large, one small) isolated on MRS were both identified as *Pediococcus pentosaceus*. The nearly full-length rRNA gene sequences (>1480 bp) for isolates DG1-12 and DG1-13 were 99.9% and 100% similar, respectively, to *P. pentosaceus* ATCC 25745 (GenBank accession No. CP000422). The entire genome sequence of *P. pentosaceus* ATCC 25745 has been recently published with that of some other LAB, all having well-documented roles in the food and feed fermentation industry (Makarova et al. 2006). Of particular interest is the ability of some *P. pentosaceus* strains to inhibit bacterial and fungal agents of spoilage by acid, bacterocin, or antifungal production (Lindgren and Dobrogosz 1990). The numbers of potentially beneficial bacteria (*P. pentosaceus*) appeared to increase over this time period relative to potential spoilage agents (aerobic heterotrophs, molds). Given the hypothesis that some LAB (i.e., lactobacilli) in wheat-based distillers grains may have probiotic potential (Pedersen et al. 2004), as well as the emerging interest in probiotics (AAM 2006) and the marketing potential for distillers grains, more detailed evaluation of LAB dynamics in corn-based DWG is needed.

Our observations of substantial culturable numbers of organisms in freshly produced DWG contrasts with the lack of detectable culturable organisms reported from freshly produced wet wheat distillers grains (a similar product) by Pedersen et al. (2004). They also documented an increase in LAB to 10^8 CFU/mL (unknown time frame, but probably <1 week), whereas our highest value for LAB was 10^6 CFU/g dry mass at 9 days. We also observed increases in aerobic heterotrophic bacteria, yeasts, and molds with time in the controlled aging of our samples, whereas Pedersen et al. (2004) only observed increases in the number of lactobacilli (no other types of bacteria) and yeasts in samples collected from different locations spanning different time intervals. In a 1983 study of corn-based DWG and control of spoilage (Nofsinger et al. 1983), high viable numbers were observed at the initial time point: about 10^7 CFU/g aerobic heterotrophic bacteria, 10^6 CFU/g yeasts and molds, and 10^6 CFU/g lactobacilli. After 10 days of aging, the numbers for all 3 categories were $\sim 10^9$ CFU/g; however, the distillers grains used in this study were produced under conditions substantially different from that of contemporary fuel ethanol plants, and it is unclear how long the grains were stored at 1 °C prior to the first analyses. The disparity between the microbiology of freshly produced and aged DWG in this study and that of other DWG (Nofsinger et al. 1983; Pedersen et al. 2004) limits generalizations regarding the microbiology of DWG products. It is unclear whether the differences in microbiology are because of the substrate or to the process, which can be quite variable (Spiehs et al. 2002).

PFLA profiles

Viable microbial biomass estimates were confounded by the sample matrix in a manner previously observed in environmental samples containing vegetable oil (Greg Davis, Microbial Insights, personal communication 2006) and are therefore not reported. The PFLA profiles for sample intervals at days 0, 4, and 9 are provided for fatty acids that were above detection in any of the samples (Table 3). Spe-

Table 3. Phospholipid fatty acid (PLFA) community structure, normalized per mole-percent, of the distillers wet grains at 3 sampling points following production.

PLFA	ECL[a]	Time zero[b]	Four days[b]	Nine days[b]
16:1ω9c	15.70	0.1 (0)	0.1 (0.1)	**0.0** (0)
16:1ω7c	15.74	0.2 (0)	0.2 (0)	**0.4** (0.1)
18:1ω7c	17.77	1.3 (0)	1.3 (0)	1.3 (0)
Total monoenoics		1.6 (0)	1.6 (0.1)	1.7 (0.1)
10me17:0 (mid-chain branched saturates)	17.43	0.0 (0)	0.0 (0)	**1.2** (0.9)
14:0	14.00	0.0 (0.1)	0.0 (0)	0.0 (0.1)
16:0	16.00	27.2 (0.3)	27.2 (0.3)	26.4 (0.8)
17:0	17.00	0.1 (0)	0.1 (0)	**0.0** (0)
18:0	17.99	1.6 (0)	1.6 (0)	1.6 (0)
20:0	19.99	0.1 (0)	0.1 (0.1)	**0.0** (0)
22:0	22.00	0.0 (0)	0.0 (0)	0.0 (0)
Total normal saturates		29.1 (0.4)	28.9 (0.2)	28.0 (0.9)
18:2ω6	17.62	43.3 (0.4)	43.3 (0.2)	43.0 (0.5)
18:3ω3	17.66	1.3 (0)	1.3 (0)	**2.1** (0.3)
18:1ω9c	17.72	24.8 (0.7)	24.9 (0.4)	24.0 (0.7)
Total polyenoics		69.3 (0.4)	69.5 (0.2)	69.1 (0.9)

Note: Data for PLFA that appear to signal a change in community structure at day 9 are in bold.
[a]ECL is the equivalent chain length in carbons.
[b]Data are means with one standard deviation in parentheses, $n = 4$ independent replicates.

cific fatty acids (the normal saturates 16:0 and 18:0; the polyenoics in general, especially 18:2ω6) may be affected by this sample matrix effect (Greg Davis, Microbial Insights, personal communication 2006). The fatty acid profiles at day 0 and day 4 were essentially unchanged, supporting the other measures that indicate the DWG were microbiologically stable over this time period. The late sampling event (day 9) was included to determine what organisms will ultimately be responsible for spoilage. Examination of the fatty acid profile for day 9, compared with the earlier samples, indicated changes in the percentage of several fatty acids (keeping in mind the actual percentage is likely a higher number owing to the sample matrix effects discussed above). Two of the monoenoic fatty acids that often represent Gram-negative *Proteobacteria* (Pinkart et al. 2002) headed in different directions, with 16:1ω9c disappearing and 16:1ω7c increasing, the latter probably reflecting a general growth of bacteria. The mid-chain branched saturate, 10me17:0, which was completely absent through day 4, increased its proportion at day 9. This fatty acid likely indicates the growth of Actinomycetes (such as the *M. luteus* cultured from the DWG) either present at low numbers in the produced grains, or more likely, introduced from blowing soil. Two of the normal saturates (17:0 and 20:0) decreased below detection in the day 9 sample, and one of the polyenoics, 18:3ω3, increased. The increase in this latter eukaryotic fatty acid probably reflects growing numbers of molds. No terminally branched saturated fatty acids that are considered markers for *Firmicutes* (including LAB) or branched monoenoic acids were detected in any of the samples. Although sample matrix effects limit more quantitative handling of the PLFA data, it is clear that the relatively stable community composition during the first 4 days was disrupted at the day 9 sampling, and that particular fatty acids that may warrant attention have been determined.

Conclusions

The rising production of distillers grains associated with fuel ethanol production and the influence of distillers grains on the energy, environmental, and economic budget of this process (Farrell et al. 2006) necessitate basic information on their microbiology. The matrix of the DWG produced severe technical difficulties for several of the culture-independent community-level analyses, total cells and PLFA. The freshly produced DWG examined in this study were not nearly as sterile as indicated by data on wet wheat distillers grains (Pedersen et al. 2004), but under the conditions of this study were microbiologically stable over at least a 4 day period. Numbers of potentially beneficial bacteria (*P. pentosaceus*) appeared to increase over this time period relative to potential spoilage agents (aerobic heterotrophs, molds). Several molds that are capable of producing mycotoxins (*Fusarium*, *Alternaria*, and *Penicillium*) colonized the DWG and grew to high densities by 9 days under moderate temperatures following production. It remains unknown how the microbial composition of distillers grains may influence non-feed uses for distillers grains.

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